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(71) Applicant: **Japan Science and Technology Corporation**
Kawaguchi-shi, Saitama 332-0012 (JP)

(72) Inventors:
- **Akira, Shizuo**
  Minoo-shi, Osaka 562-0031 (JP)
- **Kawai, Taro**
  Nishinomiya-shi, Hyogo 663-8136 (JP)

(74) Representative: **Kremer, Simon Mark et al**
Mewburn Ellis,
York House,
23 Kingsway
London WC2B 6HP (GB)

(54) DNA coding for serine/threonine kinase

(57) There is provided a DNA coding for a serine/threonine kinase.

Thus, the present invention provides a DNA coding for a protein (a) or (b):

(a) a protein comprising the amino acid sequence as shown in SEQ ID NO: 1;
(b) a protein comprising an amino acid sequence having one or several amino acids deleted, substituted or added in the amino acid sequence as shown in SEQ ID NO: 1, and exhibiting a serine/threonine kinase activity.

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Description

BACKGROUND OF THE INVENTION

Field of the Invention:

[0001] The present invention relates to a serine/threonine kinase, a DNA coding for said kinase, a recombinant vector comprising said DNA, a transformant transformed with said vector, and a process for preparing the serine/threonine kinase.

Prior Art:

[0002] Various signals from the exterior of a cell are transmitted through receptors on the cell surface into the cell and ultimately into the necleus. The signals transmitted into the nucleus activate transcription factors and, as a result, expression of a group of genes is induced or repressed to produce phenotypes such as cell proliferation, differentiation and cell death. Many transcription factors have been cloned and the structure of functional domains have been elucidated: MOLECULAR BIOLOGY OF THE CELL THIRD EDITION, pp. 401-469. These functional domains are known to include leucine zipper, helix-loop-helix and zinc finger structures. Among them, the leucine zipper structure is a motif commonly found in such transcription factors as Jun/Fos, ATF/CREB and C/EBP families and these transcription factors form homo- or hetero dimers through their leucine zipper structures to control the transcription of specific genes: Hai, T. et al., Proc. Natl. Acad. Sci., USA, 88:3720-3724 (1991).

[0003] Recently, it is reported that the leucine zipper structure is also found other functional molecules than the transcription factors (Holzman, L.B. et al., J. Biol. Chem., 269:30808-30817, 1994), suggesting that the leucine zipper structure not only facilitates the binding between transcription factors but also acts generally as a protein-protein interactional domain in cells.

[0004] Therefore, identification of molecules interacting with the leucine zipper domain is considered to be useful in analyzing not only new functions of transcription factors but also functions of the leucine zipper structure in other molecules than the transcription factors.

SUMMARY OF THE INVENTION

[0005] It is an object of the present invention to provide a serine/threonine kinase, a DNA coding for said kinase, a recombinant vector comprising said DNA, a transformant transformed with said vector, and a process for preparing the serine/threonine kinase.

[0006] As a result of their eager studies based on the above described problems, the present inventors have succeeded in isolating a DNA coding for a serine/threonine kinase from cDNA libraries prepared from human placenta and mouse brain and thus completed the present invention.

[0007] Accordingly, the present invention is the following recombinant protein (a) or (b):

(a) a protein comprising the amino acid sequence as shown in SEQ ID NO: 1;
(b) a protein comprising an amino acid sequence having one or several amino acids deleted, substituted or added in the amino acid sequence as shown in SEQ ID NO: 1, and exhibiting a serine/threonine kinase activity.

[0008] Also, the present invention is the following recombinant protein (c) or (d):

(c) a protein comprising the amino acid sequence as shown in SEQ ID NO: 2;
(d) a protein comprising an amino acid sequence having one or several amino acids deleted, substituted or added in the amino acid sequence as shown in SEQ ID NO: 2, and exhibiting a serine/threonine kinase activity.

[0009] Further, the present invention is a DNA coding for said protein. The DNA include, for example, those comprising the base sequence as shown in SEQ ID NO: 3 or 4.

[0010] The present invention is also a recombinant vector comprising said DNA.

[0011] Still further, the present invention is a transformant transformed with the recombinant vector.

[0012] Finally, the present invention is a process for producing a serine/threonine kinase comprising cultivating the transformant in a culture medium and collecting the serine/threonine kinase from the resulting culture.

BRIEF DESCRIPTION OF THE DRAWINGS

[0013] The present invention will hereinbelow be described in detail with reference to the drawings attached in which:

Fig. 1 shows the results of homology search in amino acid sequence;
Fig. 2 shows the results of homology search in amino acid sequence;
Fig. 3 is an electrophoretic photograph showing the results of western blot;
Fig. 4 is a photograph (the form of an organism) showing the results of colony formation in a selective medium;
Fig. 5 is an electrophoretic photograph showing the results of northern blot;
Fig. 6 is a photograph (the form of an organism) showing the results of colony formation in a selective medium;
Fig. 7 is a photograph of NIH3T3 (the form of an organism) showing the form of apoptosis;
Fig. 8 shows the fraction of LacZ expression cells showing the form of apoptosis;
Fig. 9 is an electrophoretic photograph showing the kinase activity of ZIP-kinase; and
Fig. 10 is a photograph (the form of an organism) showing the intracellular localization of ZIP-kinase.

DESCRIPTION OF THE INVENTION

[0014] The recombinant protein according to the present invention, hereinafter also referred to as "ZIP-kinase", is a protein molecule binding to the leucine zipper domain of a transcription factor called ATF4, and has a serine/threonine kinase activity. The ZIP-kinase is a novel nuclear serine/threonine kinase having the leucine zipper structure and has an activity to induce apoptosis. ATF4 is a leucine zipper type transcription factor which binds to cAMP response element (CRE) and belongs to the ATF/CREB family.

[0015] On the other hand, the DNA according to the present invention is obtained from cDNA libraries prepared from human placenta and mouse brain by screening them using so-called yeast two-hybrid system, and codes for ZIP-kinase. Hereinafter, the DNA will also be referred to as "ZIP-kinase DNA".

[0016] The DNA according to the present invention may be cloned in the following manner:

1. Cloning of ZIP-kinase DNA

(1) Preparation of cDNA libraries from human placenta and mouse brain

[0017] Sources of mRNA may include tissues such as human placenta and mouse brain. Established cell lines from these tissues may also be used as the source.

[0018] The mRNA may be prepared by conventional procedures. For instance, total RNA may be obtained by treating the tissue or cell with a guanidine reagent, and poly (A+) RNA (mRNA) may be obtained by an affinity column method using oligo dT-cellulose or poly U-Sepharose on Sepharose 2B as a carrier, or by a batch method. Also, the poly (A+) RNA may further be fractionated by sucrose density-gradient centrifugation.

[0019] The resulting mRNA is used as a template to synthesize a single-stranded cDNA which is in turn used to synthesize a double-stranded cDNA. A recombinant plasmid is prepared from a suitable vector DNA and used to transform Escherichia coli or the like to yield a cDNA library.

[0020] Alternatively, the cDNA library may be commercially available (CLONETECH).

(2) Construction of plasmid pAS2-1

[0021] From the cDNA library obtained in (1) above, a plasmid is prepared for screening for a desired clone.

[0022] Such a plasmid may be obtained by preparing a chimeric DNA by ligating a DNA coding for mouse ATF4 leucine zipper domain (amino acids 298 to 349 in the sequence of ATF4) to a DNA coding for GAL4 DNA binding domain, and ligating the chimeric DNA to bait plasmid pAS2-1.

(3) Screening

[0023] Then said plasmid is used to screen the cDNA library. In the screening, yeast two-hybrid system may be used. The yeast two-hybrid system is an experimental system capable of detecting interaction between proteins in yeast and is capable of screening the library for cDNA of a protein interacting with the desired protein (bait).

[0024] Positive clones may be selected using the growth in a selective medium free of hystidine, tryptophan or leucine and the activity of β-galactosidase.

(4) Determination of base sequence

[0025] The base sequence is determined for the resulting clone. The sequencing may be carried out by any known method such as Maxam-Gilbert method or the dideoxy method and is usually done using an automated base sequencer.

[0026] SEQ ID NOs: 1 and 2 exemplify the amino acid sequence of ZIP-kinase and the base sequence of ZIP-kinase DNA, respectively, according to the present invention. As far as a protein comprising said amino acid sequence has an activity as a serine/threonine kinase, there may be a mutation or variation of deletion, substitution and/or addition of one or several amino acids in said amino acid sequence as shown in SEQ ID NO: 1. For example, a protein having the amino acid sequence as shown in SEQ ID NO: 1 from which the first amino acid methionine has been deleted may also be included in the present invention.

[0027] Herein the serine/threonine kinase activity means an activity of transferring the terminal phosphate group of ATP to a certain amino acid (serine or threonine) of a protein. The introduction of mutation or variation may be carried out by any known method (Deng, W.P. et al., Anal. Biochem., 200: 81, 1992) or using a commercially available kit (Site-Directed Mutagenesis Kit of CLONETECH).

[0028] Once the base sequence of ZIP-kinase DNA according to the present invention is determined, ZIP-kinase DNA according to the present invention may then be obtained by chemical synthesis, or by PCR with various tissues-derived cDNA as a template, or hybridization of a DNA fragment having said base sequence as a probe.

2. Construction of recombinant vector and transformant

(1) Construction of recombinant vector

[0029] The recombinant vector of the present invention may be obtained by ligating or inserting ZIP-kinase DNA of the present invention into an appropriate vector. The vector for inserting ZIP-kinase DNA of the present invention is not particularly limited as long as it can be replicated in a host, and may include plasmid DNA, phage DNA, etc. The plasmid DNA may be prepared from E. coli or Agrobacterium by the alkali extraction (Bimboim, H.C. & Doly, J., (1979) Nucleic acid Res., 7:1513) or modified method. Further, commercially available plasmids may also be used, for example, pUC18 (Takara Shuzo), pUC19 (Takara Shuzo), pBluescript SK+ (Stratagene), pGEM-T (Promega), pT7 Blue (Novagen) and PBR322 (Takara Shuzo).

[0030] The phage DNA may include, for example, M13mp18, M13mp19, λ gt10, λ gt11, etc.

[0031] When the DNA of the present invention is inserted into a vector, the purified DNA may first be cut with a suitalbe restriction enzyme and inserted into a restriction enzyme site or multi cloning site of a suitalbe vector DNA to ligate with the vector.

[0032] The DNA of the present invention should be incorporated into a vector such that the function of the DNA can be realized. In addition to a promoter and the DNA of the present invention, the vector of the present invention may comprise a terminator, a ribosome-binding sequence and the like. The terminator may be a stop codon such as TGA, TAG or TAA and the ribosome-binding sequence may be a leader sequence.

(3) Preparation of transformant

[0033] The transformant of the present invention may be obtained by introducing the recombinant expression vector of the present invention into a host such that the desired gene can be expressed therein.

[0034] The host is not particularly limited so long as the DNA of the present invention can be expressed and may include, for example, bacteria belonging to the genus Escherichia or Bacillus, such as Escherichia coli and Bacillus subtillus; yeast, such as Saccharomyces cerevisiae and Saccharomyces pombe; animal cells, such as COS and CHO cells; and insect cells, such as Sf9.

[0035] When a bacterium, such as E. coli, is used as a host, it is preferred that the vector of the present invention is capable of autonomously replicating in said bacterium and comprises a promoter, a ribosome-binding sequence, the DNA of the present invention, and a transcription terminating sequence. The vector may also comprise a gene controlling the promoter.

[0036] For example, pET and pGEX (Pharmacia) may be used as the expression vector.

[0037] Any promoter may be used so long as the expression can be effected in the host such as E. coli. A promoter derived from E. coli or phage, such as trp, lac, PL or PR promoter, may be used. An artificially designed and modified promoter, such as T7 or T3 may also be used.

[0038] The method for introducing the recombinant vector into a bacterium is not particularly limited so long as a DNA can be introduced into a bacterium. For example, the method using calcium ion (Proc. Natl. Acad. Sci., USA, 69, 2110-2114 (1972)) and the electroporation method may be used.

[0039] When a yeast is used as a host, YEp13, YEp24 and YCp50 may be used as the expression vector. The

promoter used is not particularly limited so long as the expression in the yeast can be effected, and may include, for example, gall, ga110, heat shock protein, MF α1 and SV40 promoters.

[0040] The method for introducing the recombinant vector into a yeast is not particularly limited so long as a DNA can be introduced into a yeast, and include, for example, the electroporation method (Methods Enzymol., 194, 182-187 (1990)), the spheroplast method (Proc. Natl. Acad. Sci., USA, 84, 1929-1933 (1978)), and the lithium acetate method (J. Bacteriol., 153, 163-168 (1983)).

[0041] When an animal cell is used as a host, an expression vector, such as pcDNAI/Amp or pcDNAI (Invitrogen) is used. The promoter used may also be the early gene promoter of human cytomegalovirus.

[0042] The method for introducing the recombinant vector into an animal cell may include, for example, the electroporation method, the calcium phosphate method and the lipofection method.

[0043] The recombinant vectors of the present invention (one vector containing ZIP-kinase DNA from human placenta and another vector containing ZIP-kinase DNA from mouse brain) have been introduced into E. coli DH5, E. coli (hZIP-kinase) DH5 and E. coli (mZIP-kinase) DH5, respectively, and deposited at National Institute of Bioscience and Human-Technology (NIBH), Agency of Industrial Science and Technology, 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken 305, Japan on September 25, 1997 under Accession Nos. FERM BP-6487 and FERM BP-6488, respectively.

3. Production of ZIP-kinase

[0044] ZIP-kinase of the present invention may be obtained by cultivating the transformant in a culture medium and collecting from the resulting culture.

[0045] The transformant of the present invention may be cultivated in a culture medium by any method conventionally used to cultivate a host.

[0046] The culture medium for cultivating a transformant obtained from a microorganism such as E. coli or yeast as a host may be either a natural or synthetic medium so long as it contains a carbon source, a nitrogen source and inorganic salts which can be utilized by the microorganism and the transformant can efficiently be cultivated.

[0047] The carbon source used may include carbohydrates, such as glucose, fructose, sucrose, starch, and dextrose; organic acids, such as acetic acid and propionic acid; and alcohols, such as ethanol and propanol.

[0048] The nitrogen source which may be used includes ammonia; ammonium salts of inorganic or organic acids, such as ammonium chloride, ammonium sulfate, ammonium acetate and ammonium phosphate; other nitrogen containing compounds; peptone, meat extract, corn steep liquor, and yeast extract.

[0049] The minerals which may be used include monobasic potassium phosphate, dibasic potassium phosphate, magnesium phosphate, magnesium sulfate, sodium chloride, ferrous sulfate, manganese sulfate, copper sulfate, calcium carbonate, calcium chloride, and disodium phosphate.

[0050] The cultivation is generally carried out at 37°C for 12 to 18 hours under aerobic conditions, such as shaking culture and aerated spinner culture. During the cultivation, pH is kept at 7.0 to 7.5. The pH is adjusted with an inorganic or organic acid or alkaline solution, or carbonic acid gas.

[0051] During the cultivation, an antibiotic such as ampicillin or tetracycline may optionally be added to the medium.

[0052] When a microorganism transformed with an expression vector comprising an inducible promoter is cultivated, an inducer may be added to the medium, if necessary. For example, when a microorganism transformed with an expression vector comprising Lac promoter is cultivated, isopropyl-β-D-thiogalactopyranoside (IPTG) or the like may be added to the medium. When a microorganism transformed with an expression vector comprising trp promoter is cultivated, indole-acrylic acid (IAA) may be added to the medium.

[0053] When a transformant obtained from an animal cell as a host is cultivated, a conventional culture medium such as RPMI 1640 or DMEM medium or these media to which fetal bovine serum is added may be used.

[0054] The cultivation is generally carried out at 37°C for 1 to 3 days in the presence of 5% CO_2.

[0055] During the cultivation, an antibiotic such as kanamycin or penicillin may be added to the medium.

[0056] After the cultivation, when ZIP-kinase of the present invention is produced in the host cell, the ZIP-kinase is extracted by disruption of the cell. When ZIP-kinase of the present invention is produced in the exterior of the cell, the culture may be directly used as it is, or the ZIP-kinase of the present invention may be isolated and purified from the culture, after removing the cell by centrifugation, using any conventional biochemical methods generally used in the isolation and purification of proteins, such as ammonium sulfate precipitation, gel chromatography, ion exchange chromatography and affinity chromatography, singly or in any combination thereof.

EXAMPLES

[0057] The present invention will be further illustrated by the following examples. However, the scope of the present invention is not limited to these examples.

Example 1: Cloning of ZIP-kinase DNA

(1) Preparation of cDNA library

[0058] A commercially available cDNA (CLONETECH) was used in the present invention.

(2) Construction of plasmid

[0059] DNA coding for leucine zipper domain of mouse ATF4 was obtained by PCR method.

[0060] The composition of the PCR reaction was 1.0 μg DNA, 10 mM Tris-HC1 (pH 8.3), 50 mM KCl, 1.5 mM $Mgcl_2$, 0.2 mM dNTP, 0.5 μM primer, and 1 U Taq.

[0061] The following primers were used:

Sense primer: 5'-GGGAATTCGCGGAGCAGGAGGCT-3' (SEQ ID NO: 5)

Antisense primer: 5'-GGGGATCCCTAGGGGACCCTTTTCTA-3' (SEQ ID NO: 6)

[0062] Sense primer: 5'-GGGAATTCGCGGAGCAGGAGGCT-3' (SEQ ID NO: 5) Antisense primer: 5'-GGGGATC-CCTAGGGGACCCTTTTCTA-3' (SEQ ID NO: 6)

[0063] PCR reaction was first carried out at 94°C for 1 minute. Then, 25 cycles of reactions at 94°C for 20 seconds, at 56°C for 20 seconds and at 72°C for 30 seconds were carried out. Finally, the reaction at 72°C for 10 minutes was effected.

[0064] The PCR products were cut with EcoRI/BamHI and inserted into EcoRI/BamHI site of pAS2-1 vector. The plasmids were used to transform E. coli DH5 α and purified by means of a commercially available kit (Wizard miniprep: Promega) based on alkali-SDS method. This plasmid capable of expressing a fused protein of GAL4 DNA binding domain in yeast was used as a bait.

(3) Screening

[0065] Yeast strain Y190 was transformed with the plasmid as a bait using MATCHMAKER Two-Hybrid System kit of CLONETECH. Transformants were selected by growth in tryptophan(-) medium as an index. Further, cDNA libraries capable of expressing a fusion protein with GAL4 transcription activating domain (CLONETECH, mouse brain and human placenta MATCHMAKER cDNA libraries) were transformed. Transformants can grow in tryptophan(-), leucine (-) medium. Further, since reporter genes, HIS3 and LacZ genes, were transcribed if the bait bound to the DNA coding for the protein from the library, positive clones can grow in tryptophan (-), leucine (-), histidine (-) medium and provides blue color in the presence of X-gal because of their β -galactosidase activity. Plasmids were purified from the positive clones using MATCHMAKER Two-Hybrid System kit of CLONETECH, and used to transform E. coli. Plasmids were purified from the resulting transformants and the base sequences thereof were determined (ABI model 377). The resulting base sequences were searched for homology using GenBank, EMBL, DDBJ data base.

[0066] As a result, those having high homology (20% or higher) with the previously reported C/EBP family, AP-1 family and genes having leucine zipper structure were identified as novel genes. Seven (7) and 2 clones of such genes were obtained from mouse brain and human placenta cDNA libraries, respectively. All these genes were derived from an identical gene.

(4) Determination of base sequence

[0067] The thus obtained gene was considered to code for a kinase and the DNA coding for this novel kinase was designated as ZIP-kinase DNA (Zipper Interacting Protein Kinase DNA). The base sequence of the full length ZIP-kinase DNA was determined.

[0068] The base sequences of ZIP-kinase DNA obtained from human placenta and mouse brain are shown in SEQ ID NOs: 3 and 4, respectively. The amino acid sequences encoded by the base sequences of SEQ ID NOs: 3 and 4 are shown in SEQ ID NOs: 1 and 2, respectively.

[0069] The amino acid sequence encoded by ZIP-kinase DNA obtained from human placenta (human ZIP-kinase) and the amino acid sequence encoded by ZIP-kinase DNA obtained from mouse brain (mouse ZIP-kinase) were

searched for homology therebetween and the leucine zipper domain and serine/threonine kinase domain were found in the C- and N-terminal of the respective amino acid sequences, respectively (Fig. 1). Further, mouse and human ZIP-kinases consisted of 448 and 454 amino acids, respectively, and the homology between mouse and human was 84.9% at amino acid level.

[0070] Moreover, the kinase domains of the ZIP-kinases showed high homology with DAP-kinases positively controlling apoptosis caused by IFN-γ, suggesting that these kinases form a new family (Fig. 2).

[0071] Example 2: Construction of recombinant vector and preparation of transformant

[0072] To construct a recombinant vector of ZIP-kinase DNA, cDNA coding for ZIP-kinase was synthesized by PCR method.

[0073] The PCR reaction mixture and primers used were as follows.

[0074] The composition of the PCR reaction was 1.0 μg DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.5 μM primer, and 1 U Taq.

Sense primer: 5'-GGGTCGACCAC CATGGCTTAC CCATACGATG TTCCAGATTA CGCTATGTCC ACATTCAGGC AA-3' (SEQ ID NO: 7)

Antisense primer: 5'-GGGTCGACTA GCGCACGCCG CACTCAGCCT GC-3' (SEQ ID NO: 8)

[0075] PCR reaction was first carried out at 96°C for 1 minute. Then, 30 cycles of reactions at 96°C for 30 seconds, at 56°C for 30 seconds and at 72°C for 1 minute were carried out. Finally, the reaction at 72°C for 10 minutes was effected.

[0076] The resulting PCR products were cut with SalI, inserted into expression vector pEF-BOS (Takara, Ligation kit), and used to transform E. coli DH5 (TOYOBO). Plasmids were purified from the E. coli (Promega, Wizard miniprep) and confirmed by DNA sequence (ABI, model 377).

[0077] A DNA coding for a variant of ZIP-kinase in which the 42nd amino acid lysine in the amino acid sequence as shown in SEQ ID NO: 2 was changed to alanine, hereinafter referred to as "ZIP-kinase K42A", was constructed by using Site-Directed Mutagenesis Kit of CLONETECH. Also, a DNA coding for another variant in which the 422nd and 429th amino acids valine and the 436th amino acid leucine in the amino acid sequence as shown in SEQ ID NO: 2 were changed to alanines, hereinafter referred to as "ZIP-kinase LA", was similarly constructed.

Example 3: Function of DNA of the present invention

(1) Binding of ZIP-kinase to ATF4 in cells

[0078] Whether ZIP-kinase binds to ATF4 in cells as well or not was investigated.

[0079] First, DNA coding for mouse ZIP-kinase (309-448 amino acids in the amino acid sequence as shown in SEQ ID NO: 2) was inserted into expression vector pEF-BOS. Thus, a tag of the transcription factor Myc was provided at the N-terminal end of ZIP-kinase, whereby a DNA coding for Myc-ZIP-kinase complex was designated to have the tag as an epitope to construct the vector (pEF-BOS-Myc-ZIP-kinase). Also, an expression vector (pEF-BOS-FLAG-ATF4) was constructed comprising a DNA coding for human ATF4 (full length)-FLAG complex in which FLAG epitope had been added to the N-terminal end of human ATF4.

[0080] These vectors were transiently introduced into COS-7 cell line by the lipofection method and expressed (Fig. 3 in which lanes 1, 4, 7 and 10 represent FLAG-ATF4; lanes 2, 5, 8 and 11 Myc-ZIP-kinase; lanes 3, 6, 9 and 12 FLAG-ATF4 and Myc-ZIP-kinase). 36 hours after the introduction, the cells were collected and solubilized with 0.5% Nonidet P-40 lysis buffer. The resulting solubilized cell (WCE: whole cell extract) was developed in SDS-PAGE, and transferred to nitrocellulose membrane. Western blot analysis was done using anti-FLAG monoclonal antibody (Fig. 3, lanes 1, 2 and 3) and anti-Myc monoclonal antibody (lanes 7, 8 and 9) to confirm the expression of Myc-ZIP-kinase and FLAG-ATF4.

[0081] Subsequently, the WCE was immunoprecipitated with anti-Myc monoclonal antibody and the precipitate was subjected to the western blot analysis using anti-FLAG monoclonal antibody, attempting to detect co-immunoprecipitation of Myc-ZIP-kinase and FLAG-ATF4 (Fig. 3, lanes 4 to 6).

[0082] As a result, a band of FLAG-ATF4 was detected in lane 6 (Fig 3) . For further confirmation, the WCE was then immunoprecipitated with anti-FLAG monoclonal antibody and the precipitate was subjected to the western blot analysis

using anti-Myc monoclonal antibody (lanes 10, 11 and 12). A band of Myc-ZIP-kinase immunoprecipitated with FLAG-ATF4 was found only in lane 12.

[0083] Thus, it was shown that ZIP-kinase and ATF4 bind to each other in cells as well.

[0084] From this result that ZIP-kinase and ATF4 binds to each other, it may be considered that ATF4 may possibly control the activity of ZIP-kinase.

(2) Determination of domain necessary for binding of ZIP-kinase to ATF4

[0085] The site to which ZIP-kinase and ATF4 bind was determined using yeast two-hybrid system. First, variants of mouse ZIP-kinase were prepared: 1) amino acids 278 to 448 of ZIP-kinase (ZIP-kinase 278-448); 2) leucine zipper domain of ZIP-kinase (amino acids 398 to 448) (ZIP-kinase LZ); and 3) a variant of ZIP-kinase in which valine and leucine in the leucine zipper domain were substituted with alanine (ZIP-kinase LA). Each of these variants was designed to produce a chimeric protein with GAL4 trans activating domain, and DNA coding for said chimeric protein was inserted into pACT2 and introduced into yeast strain Y190 together with pAS2-1-ATF4 LZ. The strain was cultivated on histidine+, tryptophan-, leucine-, and histidine-, tryptophan-, leucine- selective media.

[0086] Yeast containing DNA coding for ZIP-kinase 278-448 and yeast containing DNA coding for ZIP-kinase LZ could form a colony on the histidine-, tryptophan-, leucine- medium, indicating that ZIP-kinase bound to ATF4 through leucine zipper domain present at the C-terminal (Fig. 4) . Further, when valine and leucine in the leucine zipper domain structure were substituted with alanine, the binding to ATF4 was no longer found.

[0087] Accordingly, it has been elucidated that ZIP-kinase and ATF4 bind to each other through their respective leucine zipper domain.

(3) Expression of ZIP-kinase in each tissue

[0088] Northern blot analysis was carried out to investigate the expression of ZIP-kinase in each tissue.

[0089] As shown in Fig. 5, mRNA of ZIP-kinase (about 1.4 kb) was distributed almost all tissues investigated. However, only low expression was observed in the spleen.

(4) Confirmation of formation of homodimer of ZIP-kinase

[0090] The leucine zipper domain present at the C-terminal of ZIP-kinase is considered to be a domain through which proteins bind to each other. Whether ZIP-kinase forms a homodimer or not was investigated. Plasmid pAS2-1 into which DNA coding for leucine zipper domain of ZIP-kinase was inserted, and plasmid pACT2 into which DNA coding for the leucine zipper domain of ZIP-kinase and a variant in which valine and leucine in said domain were substituted with alanine, were co-introduced into yeast and colony formation was observed in a selective medium.

[0091] As shown in Fig. 6, only yeast co-expressing the ZIP-kinase leucine zipper domain could grow in histidine-, tryptophan-, leucine- medium. Thus, it has been elucidated that ZIP-kinase forms a homodimer through its leucine zipper structure.

(5) Induction of apoptosis by ZIP-kinase

[0092] It has been shown that DAP-kinase, which has high homology with kinase domain of ZIP-kinase, induces apoptosis in HeLa cell. Whether ZIP-kinase has an apoptosis activity was investigated.

[0093] RNA wild type ZIP-kinase tagged with HA (pEF-BOS-HA-ZIP-kinase), a variant thereof in which lysine (42nd amino acid), which is present in ZIP-kinase subdomain II and conserved in other kinases, was substituted with alanine (pEF-BOS-HA-ZIP-kinase K42A), and a variant in which valine and leucine in the leucine zipper domain were substituted with alanine (pEF-BOS-HA-ZIP-kinase LA) were prepared, and DNA coding for each of these proteins was transiently introduced into NIH 3T3 cell together with LacZ expression vector (pEF-BOS-LacZ). After 36 hours from the introduction, X-gal staining was effected.

[0094] As a result, a form of cells stained blue was observed under a micorscope (Fig. 7). As compared with a control pEF-BOS-mock (Fig. 7, left, upper), the cell into which the wild type ZIP-kinase was introduced (Fig. 7, right, upper) exhibited a typical form of apoptosis associated with agglomeration of nucleus. The fraction of LacZ expression cells showing the apoptosis form was measured to be 44.9% (Fig. 8).

[0095] On the other hand, such change of form was not observed in the ZIP-kinase-K42A variant (Fig. 7, left, lower) and there was no significant difference in the fraction of apoptosis between the vaiant and control. Further, in the ZIP-kinase-LA (Fig. 7, right, lower), some cells caused apoptosis but the fraction thereof was significantly reduced as compared with the wild type

[0096] From the above results, the kinase activity of ZIP-kinase is considered to be essential for the induction of

apoptosis by the expression of ZIP-kinase. Further, since apoptosis was suppressed in vatiants in which a homodimer between ZIP-kinases was inhibited, it is suggested that ZIP-kinases form a homodimer to become an activated form.

(6) Kinase activity of ZIP-kinase

[0097] Whether ZIP-kinase indeed has an activity as a kinase was investigated.

[0098] Each of pEF-BOS-HA-ZIP-kinase, pEF-BOS-HA-ZIP-kinase K42A, and pEF-BOS-HA-ZIP-kinase LA was transiently introduced into COS-7 cell, and 36 hours later, the cell was collected and solubilized with 0.5% Nonidet P-40 lysis buffer. The solubilized cell was immunoprecipitated with anti-HA monoclonal antibody and the kinase activity in the precipitate was detected by in vitro kinase assay (Fig. 9A).

[0099] As a result, a band of phosphorylation by ZIP-kinase was observed at about 50 kDa in the wild type ZIP-kinase (Fig. 9A, lane of HA-ZIP-kinase), while no band corresponding thereto was observed in ZIP-kinase K42A. On the other hand, a phosphorylation band was observed in ZIP-kinase LA. However, when the expression of HA-ZIP-kinase and its variant in the solubilized cell was checked by the western blotting using anti-HA monoclonal antibody, the amount of HA-ZIP-kinase expressed was markedly reduced as compared with the other two variants (Fig. 9B). From this result, it may be considered that the kinase activity observed in ZIP-kinase LA would be very weak as compared with the wild type.

[0100] Further, the amount of wild type ZIP-kinase expressed was low in COS-7 cells; this is considered to be resulted from some lethal effect, such as apoptosis, of ZIP-kinase on COS-7 cells.

(7) Localization of ZIP-kinase in cells

[0101] Knowledge of intracellular localization of ZIP-kinase would be considered to be very effective in analyzing the functions of ZIP-kinase. The present inventors have investigated the localization of ZIP-kinase using a confocal laser microscope.

[0102] An expression vector (pEF-BOS-FLAG-ATF4) comprising DNA coding for ATF4 tagged with FLAG or another vector (pEF-BOS-FLAG-ZIP-kinase K42A) coding for ZIP-kinase K42A tagged with FLAG was transiently introduced into COS-7 cells. After 36 hours, the cells were fixed, reacted with anti-FLAG monoclonal antibody, and stained using FITC-labelled anti-mouse immunoglobulin antibody as a secondary antibody.

[0103] When observed under the confocal laser microscope, the cytoplasm was not stained and the nucleus was stained in the FLAG-ATF4 introduced cells (Fig. 10A). When the localization of FLAG-ZIP-kinase was similarly investigated, the same staining pattern as in ATF4 was observed, confirming that it was localized in the nucleus (Fig. 10B).

[0104] Accordingly, it could be concluded that the ZIP-kinase is a novel nuclear serine/threonine kinase.

Advantages of the Invention:

[0105] According to the present invention, there are provided a serine/threonine kinase, a DNA coding for said kinase, a recombinant vector comprising said DNA, and a transformant transformed with said vector, and a process for the preparation of the serine/threonine kinase.

[0106] Since the ZIP-kinase has a function of inducing apoptosis, the ZIP-kinase and DNA coding for said kinase are useful in being utilizable as a gene therapeutical agent against a cancer and as an anti-cancer agent.

Annex to the description

[0107]

SEQUENCE LISTING

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<151> 1997-09-26

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Tyr Leu His Ser Lys Arg Ile Ala His Phe Asp Leu Lys Pro Glu Asn
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Val Ser Ile Leu Arg Glu Ile Arg His Pro Asn Ile Ile Thr Leu His
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Ser Trp Ile Lys Val Arg Arg Arg Glu Asp Gly Ala Arg Lys Pro Glu
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His Ser Ser Met Pro Arg Asn Thr Ser Tyr Ala Ser Phe Glu Arg Phe
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gca gcc aag ttc atc aag aag cgc cgc ctg tca tcc agc cgg cgt ggg 258

Ala Ala Lys Phe Ile Lys Lys Arg Arg Leu Ser Ser Ser Arg Arg Gly

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Ile Glu Ala Gly Asn Glu Phe Lys Asn Ile Phe Gly Thr Pro Glu Phe
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Claims

1. A recombinant protein (a) or (b):

   (a) a protein comprising the amino acid sequence as shown in SEQ ID NO: 1;
   (b) a protein comprising an amino acid sequence having one or several amino acids deleted, substituted or added in the amino acid sequence as shown in SEQ ID NO: 1, and exhibiting a serine/threonine kinase activity.

2. A recombinant protein (c) or (d):

   (c) a protein comprising the amino acid sequence as shown in SEQ ID NO: 2;
   (d) a protein comprising an amino acid sequence having one or several amino acids deleted, substituted or added in the amino acid sequence as shown in SEQ ID NO: 2, and exhibiting a serine/threonine kinase activity.

3. A DNA coding for a protein (a) or (b):

   (a) a protein comprising the amino acid sequence as shown in SEQ ID NO: 1;
   (b) a protein comprising an amino acid sequence having one or several amino acids deleted, substituted or added in the amino acid sequence as shown in SEQ ID NO: 1, and exhibiting a serine/threonine kinase activity.

4. A DNA coding for a protein (c) or (d):

   (c) a protein comprising the amino acid sequence as shown in SEQ ID NO: 2;
   (d) a protein comprising an amino acid sequence having one or several amino acids deleted, substituted or added in the amino acid sequence as shown in SEQ ID NO: 2, and exhibiting a serine/threonine kinase activity.

5. The DNA of claim 3 comprising the base sequence as shown in SEQ ID NO: 3.

6. The DNA of claim 4 comprising the base sequence as shown in SEQ ID NO: 4.

7. A recombinant vector comprising a DNA of any one of claims 3 to 6.

8. A transformant transformed with a recombinant vector of claim 7.

9. A process for producing a serine/threonine kinase comprising cultivating a transformant of claim 8 in a culture medium and collecting the serine/threonine kinase from the resulting culture.

FIG. 1

Protein kinase domain
mouse ZIP-kinase MSTFRQEDVEDHYEMGEELGSGQFAIVRKCQQKGTGMEYAAKFIKKRRLPSSRRGVSREE 60
human ZIP-kinase MSTFRQEDVEDHYEMGEELGSGQFAIVRKCRQKGTGKEYAAKFIKKRRLSSSRRGVSREE 60
mouse ZIP-kinase IEREVSILREIRHPNIITLHDVFENKTDVVLILELVSGGELFDFLAEKESLTEDEATQFL 120
human ZIP-kinase IEREVNILREIRHPNIITLHDIFENKTDVVLILELVSGGELFDFLAEKESLTEDEATQFL 120
mouse ZIP-kinase KQILDGVHYLHSKRIAHFDLKPENIMLLDKHAASPRIKLIDFGIAHRIEAGSEFKNIFGT 180
human ZIP-kinase KQILDGVHYLHSKRIAHFDLKPENIMLLDKNVPNPRIKLIDFGIAHKIEAGNEFKNIFGT 180
mouse ZIP-kinase PEFVAPEIVNYEPLGLEADMWSIGVITYILLSGASPFLGETKQETLTNISAVNYDFDEEY 240
human ZIP-kinase PEFVAPEIVNYEPLGLEADMWSIGVITYILLSGASPFLGETKQETLTNISAVNYDFDEEY 240
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human ZIP-kinase FSNTSELAKDFIRRLLVKDPKRRMTIAQSLEHSWIKAIRRRNVRGEDSGRKPERRRLKTT 300
mouse ZIP-kinase RLREYSLKSHSSMPRNTSYASFERFSRVLEDVAAAEQGLRELQRGRRQCRERVCALRAAA 355
human ZIP-kinase RLKEYTIKSHSSLPPNNSYADFERFSKVLEEAAAAEEGLRELQRSRRLCHEDVEALAAIY 360
mouse ZIP-kinase EQREARCRDGSAGLGRDLRRLRTELGRTEALRTRAQEEARAALLGAGGLKRRLCRLENRY 415
human ZIP-kinase EEKEAWYREESDSLGQDLRRLRQELLKTEALKRQAQEEAKGALLGTSGLKRRFSRLENRY 420
Leucine zipper domain
mouse ZIP-kinase DALAAQVAAEVQRVRDLVRALEQERLQA-ECGVR 448
human ZIP-kinase EALAKQVASEMRRVQDLVRALEQEKLQGVECGLR 454

FIG. 2

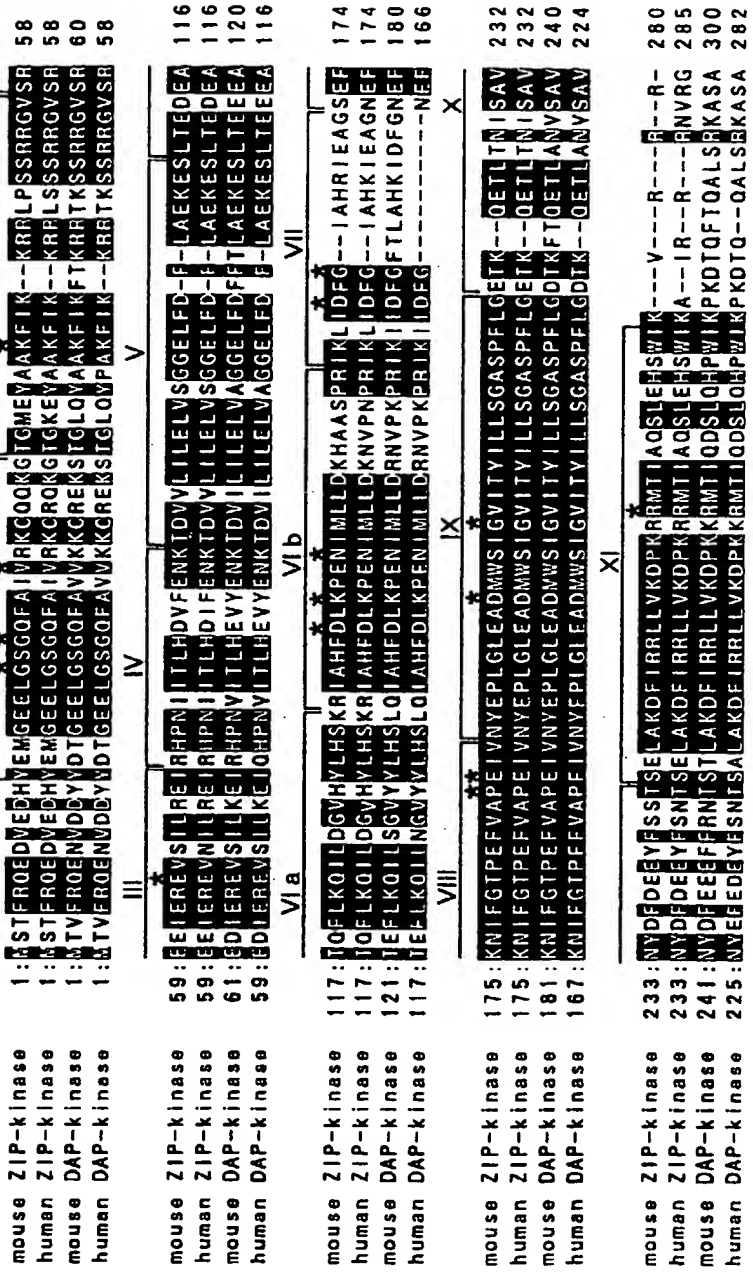

FIG. 3

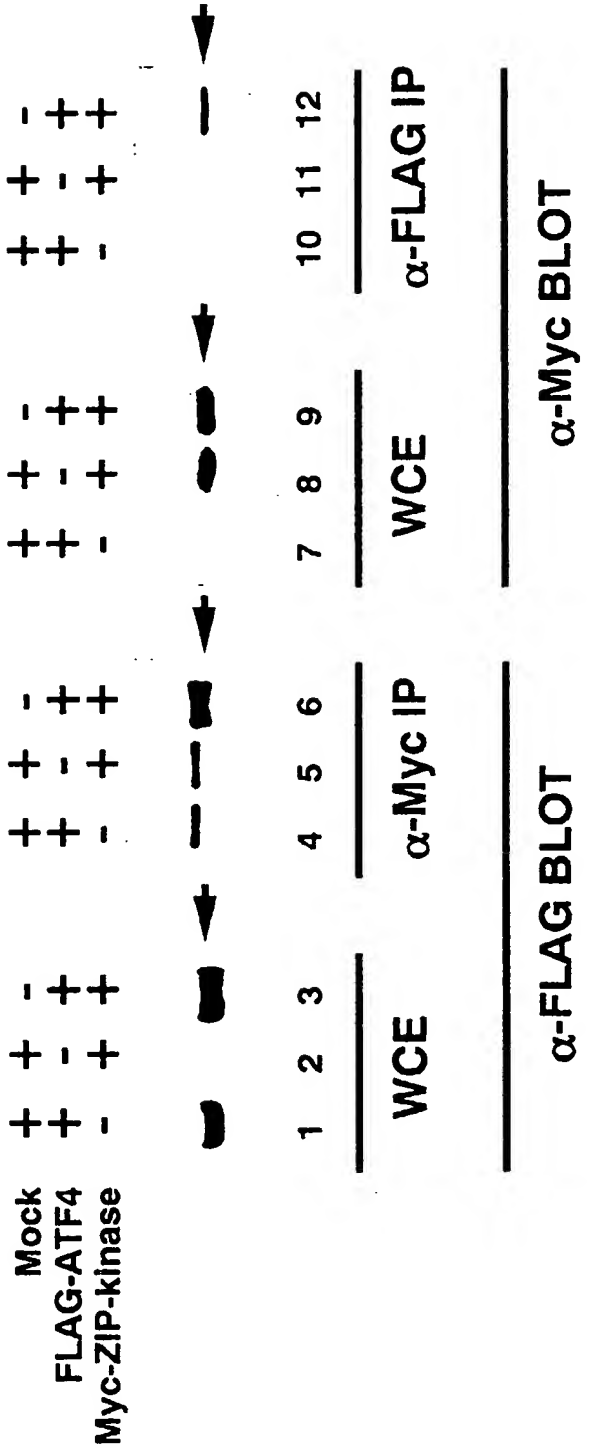
Mock
FLAG-ATF4
Myc-ZIP-kinase
1 2 3 4 5 6 7 8 9 10 11 12
WCE
α-Myc IP
WCE
α-FLAG IP
α-FLAG BLOT
α-Myc BLOT

FIG. 4

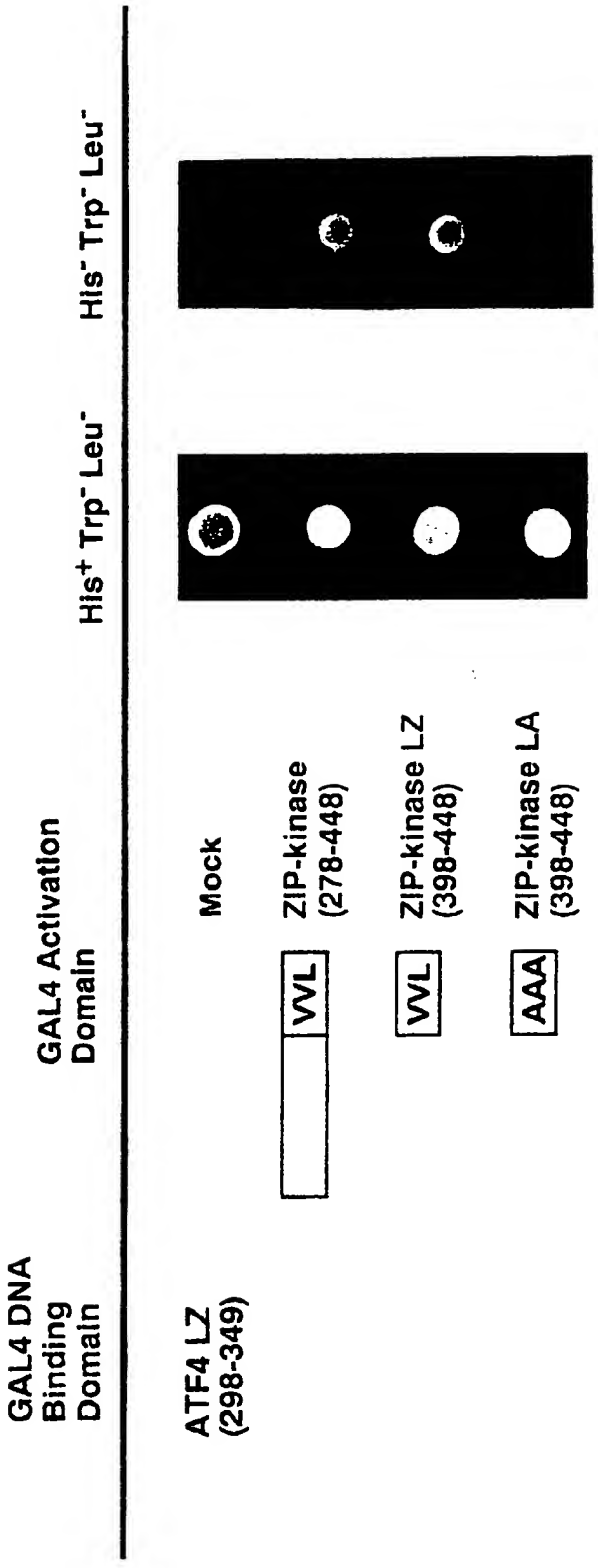
GAL4 DNA Binding Domain
GAL4 Activation Domain
His+ Trp- Leu-
His- Trp- Leu-
ATF4 LZ (298-349)
Mock
ZIP-kinase (278-448)
VVL
ZIP-kinase LZ (398-448)
VVL
ZIP-kinase LA (398-448)
AAA

FIG. 5

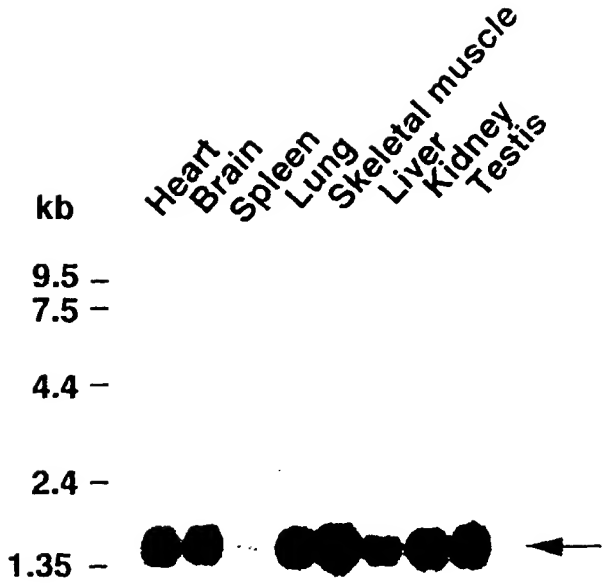
Heart
Brain
Spleen
Lung
Skeletal muscle
Liver
Kidney
Testis
kb
9.5
7.5
4.4
2.4
1.35

FIG. 6

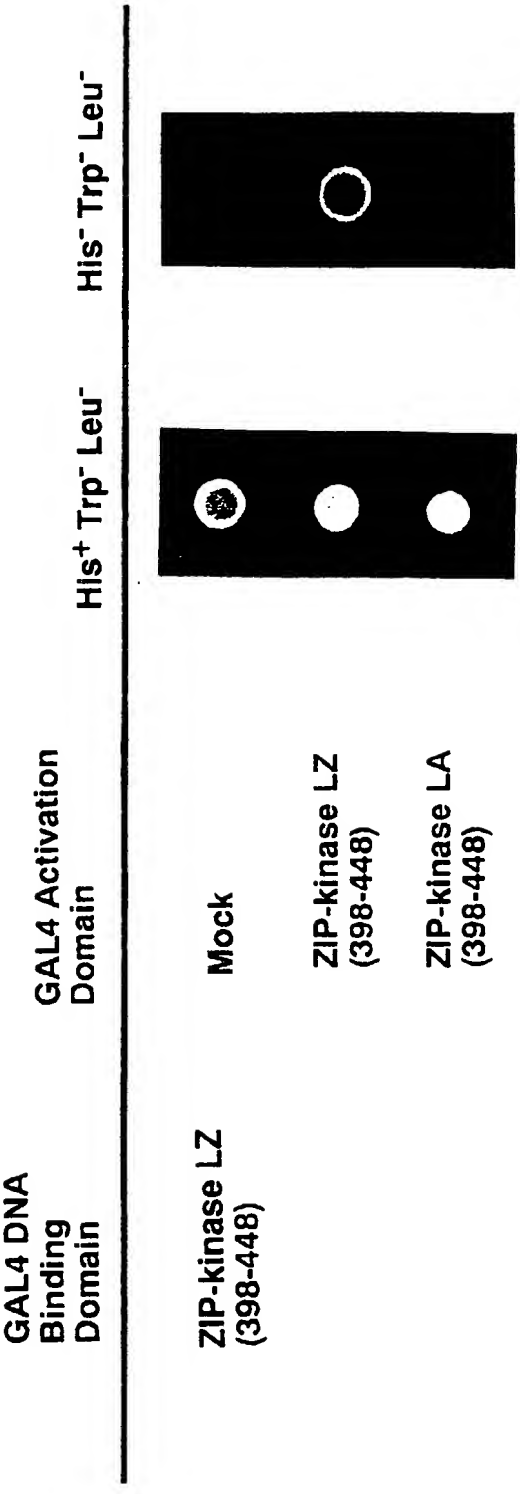
GAL4 DNA Binding Domain
GAL4 Activation Domain
His+ Trp- Leu-
His- Trp- Leu-
ZIP-kinase LZ (398-448)
Mock
ZIP-kinase LZ (398-448)
ZIP-kinase LA (398-448)

FIG. 7

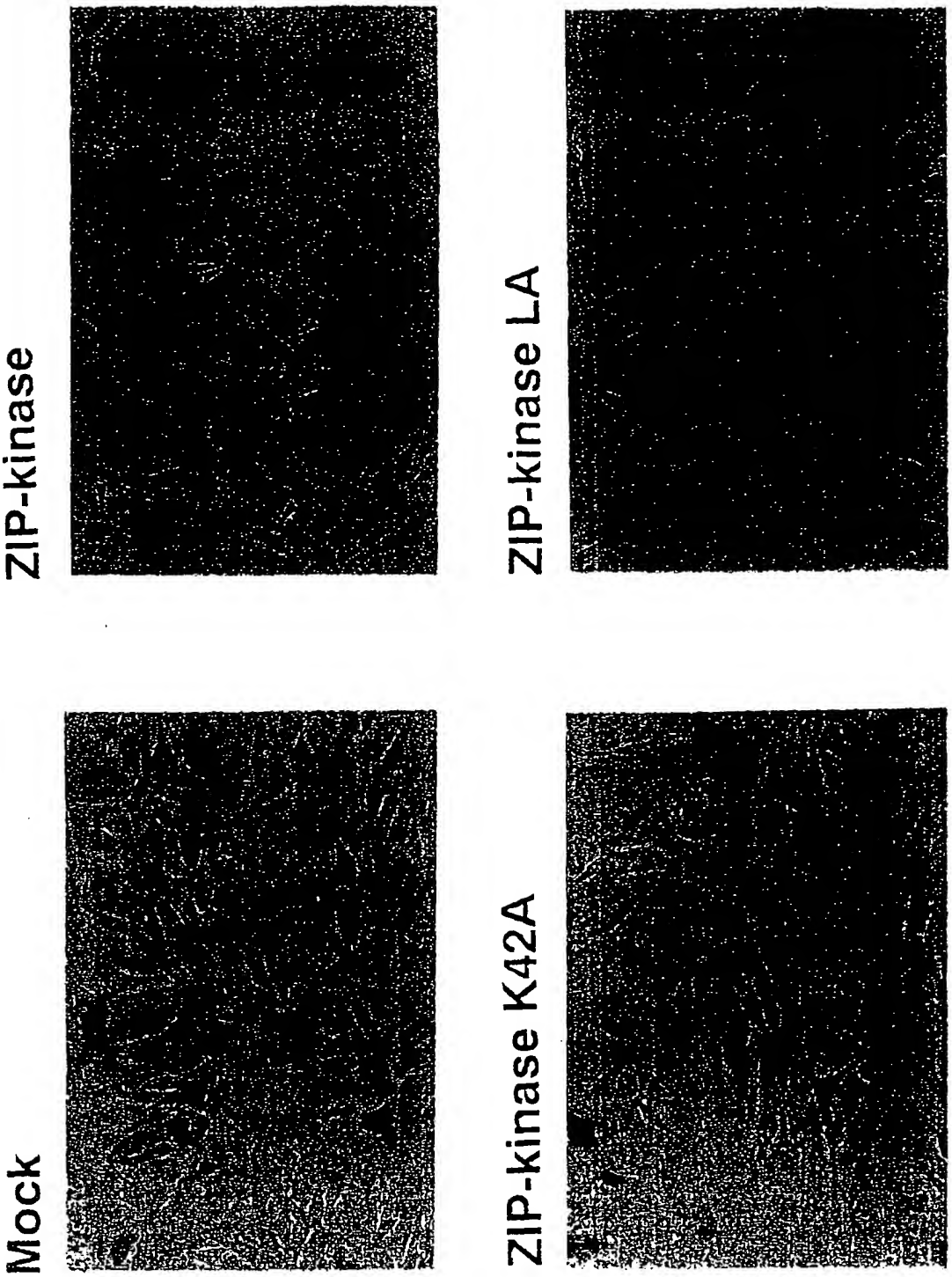

FIG. 8

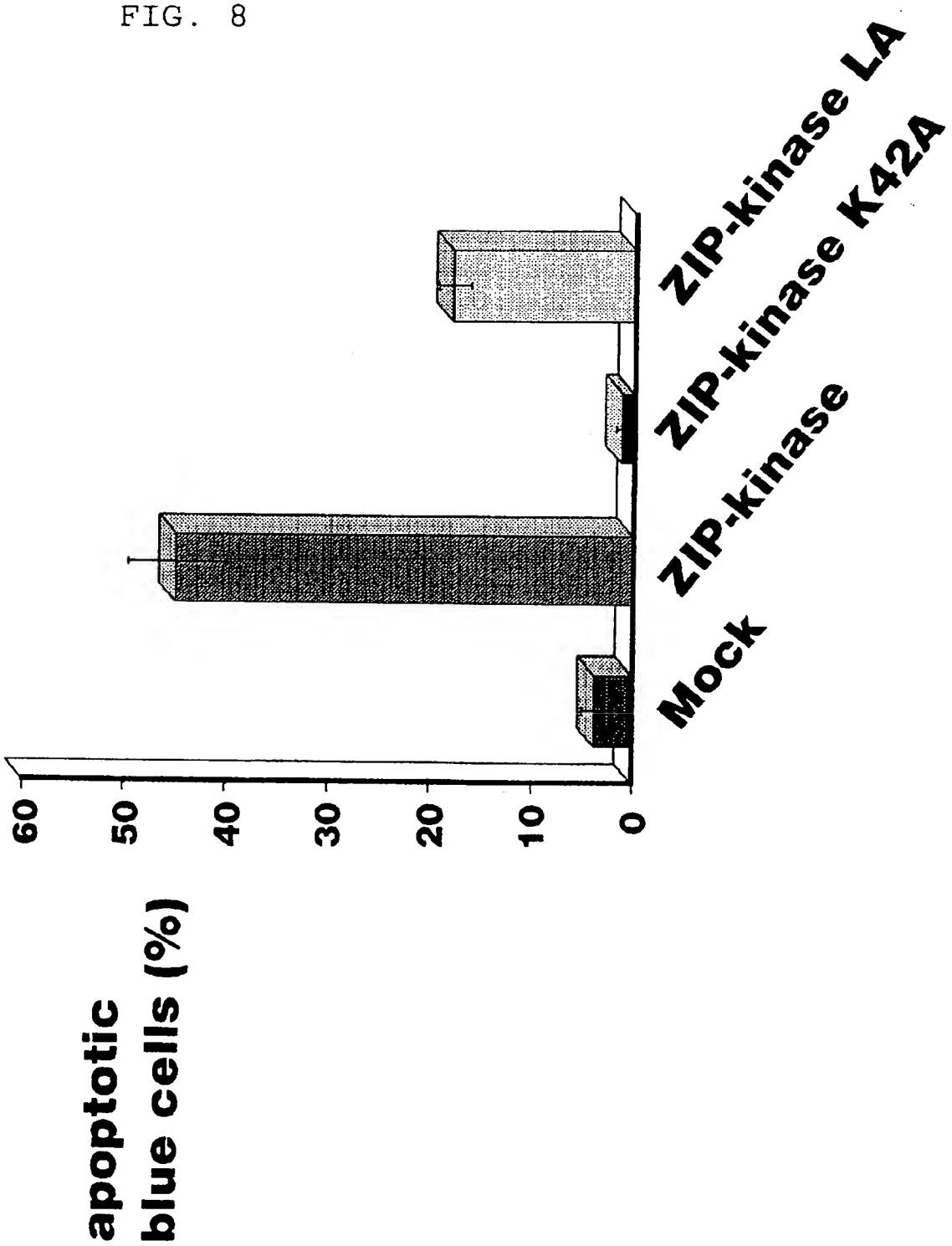
apoptotic
blue cells (%)
60
50
40
30
20
10
0
Mock
ZIP-kinase
ZIP-kinase K42A
ZIP-kinase LA

FIG. 9A

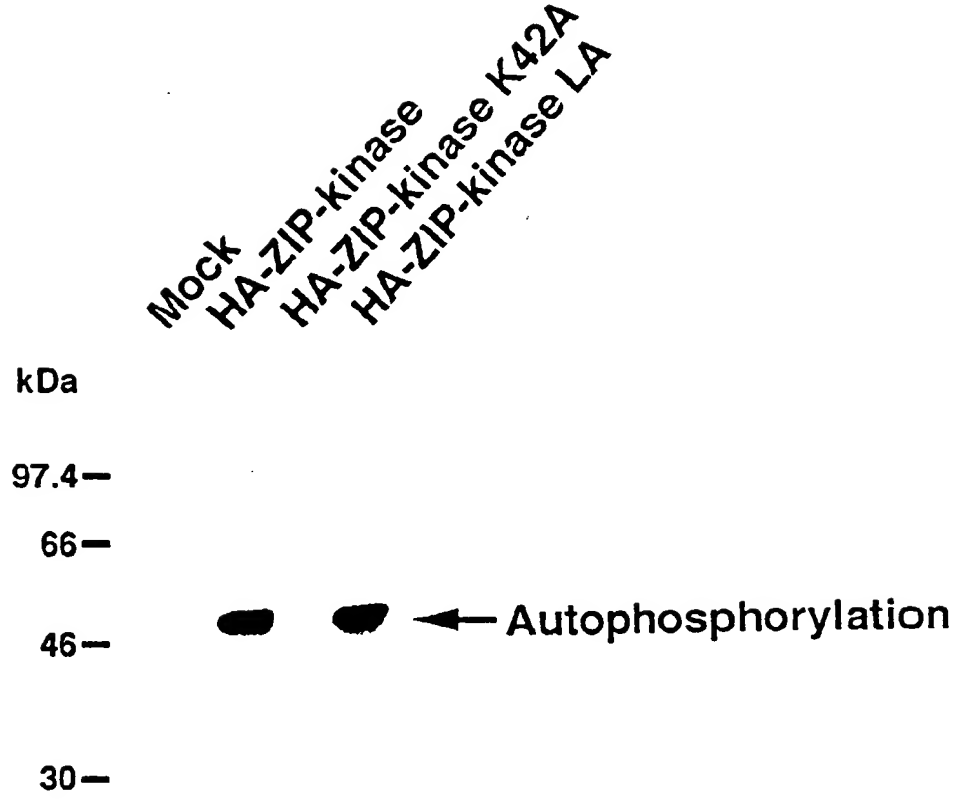
Mock
HA-ZIP-kinase
HA-ZIP-kinase K42A
HA-ZIP-kinase LA
kDa
97.4
66
46
30
Autophosphorylation

FIG. 9B

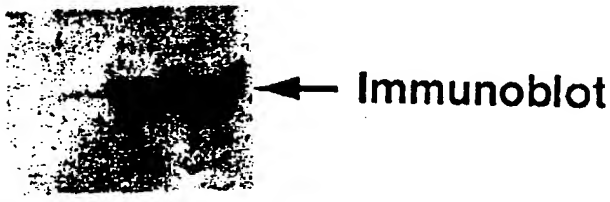
Immunoblot

FIG. 10A

FLAG-ATF4

FIG. 10B

FLAG-ZIP-kinase K42A